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ORIGINAL ARTICLE

Expression of protease-activated receptor-1 and -2 in orofacial granulomatosis

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OBJECTIVE: Orofacial granulomatosis (OFG) is a rare condition characterized by non-caseating granulomas in the orofacial region. Protease-Activated Receptors (PARs) play a role in inflammatory diseases in diverse human tissues. The aim of the study was to investigate the expression of PAR-1, PAR-2, MMP-2, MMP-9, COX-1, and COX-2 in tissues taken from OFG patients.

METHODS: PAR-1, PAR-2, MMP-2, MMP-9, COX-1, and COX-2 expression was evaluated by immunohistochemistry in biopsies taken from oral Crohn's disease (five cases), Melkersson–Rosenthal syndrome (MRS) (six cases), cheilitis granulomatosa (five cases) and normal oral mucosa (five cases).

RESULTS: PAR-1 was observed in mononuclear inflammatory cells in edematous/lichenoid lesions, whereas a strong PAR-2 immunostaining was detected in epithelioid histiocytes and giant cells in granulomatous lesions, irrespective of the clinical features (Crohn *vs* MRS). MMPs and COX-2 were expressed in the inflammatory component of edematous/lichenoid lesions and markedly overexpressed in granulomatous lesions. COX-1 was weakly and variably expressed in both edematous/lichenoid and granulomatous lesions.

CONCLUSION: Thus, PAR-1 and PAR-2 expressions were related to the intensity and type of inflammatory response but not to the type of clinical lesion. Simultaneous overexpression of PARs, MMPs and COXs suggests synergism among these proinflammatory receptors and enzymes.
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Keywords: orofacial granulomatosis; PAR-1; PAR-2; MMP-2; MMP-9; COX-1; COX-2; immunohistochemistry

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Introduction

The term 'orofacial granulomatosis' (OFG) was originally introduced to encompass a broad spectrum of non-caseating granulomatous inflammatory conditions, affecting the oral and maxillo-facial region, including Melkersson–Rosenthal syndrome (MRS), cheilitis granulomatosa (CG), sarcoidosis, oral Crohn's disease (OCD) and some infectious disorders (Weisenfeld *et al*, 1985; Ficarra *et al*, 1993; van der Waal *et al*, 2002). More recently, the term OFG has been more restrictively used to indicate only the idiopathic forms, e.g. MRS and CG (Mignogna *et al*, 2003; Sciubba and Said-Al-Naief, 2003), thereby excluding oral granulomatous inflammation because of systemic or local diseases (Crohn's disease, sarcoidosis, infectious disorders, foreign body reactions and food and contact allergies). Patients with MRS only occasionally (8–25%) express the classical clinical triad of orofacial swelling, fissured tongue and facial paralysis (Sciubba and Said-Al-Naief, 2003). More commonly, MRS has a mono-symptomatic or oligo-symptomatic clinical presentation (Daoud and Rogers, 1995; Rogers, 1996; Sciubba and Said-Al-Naief, 2003). CG is a monosymptomatic form of MRS and is clinically characterized by persistent, painless labial enlargement (Rogers, 1996; Sciubba and Said-Al-Naief, 2003).

Review of the literature shows that the relationship between OCD and OFG is a more controversial and still unresolved issue, as there have been reports of OFG being the initial presentation of OCD, as well as many examples of OCD eventually developing bowel involvement (Girlich *et al*, 2002; Bogenrieder *et al*, 2003; van de Scheur *et al*, 2003; Leão *et al*, 2004). Hence, classification and management of OFG patients requires routine investigations in search of possible gastrointestinal involvement.

Recently, considerable interest has been paid to the role of protease-activated receptors (PARs) in pathophysiology. PARs are a novel family of the G-protein

coupled receptors that require proteolytic cleavage of their amino-terminal domain to be activated (MacFarlane *et al*, 2001; Ossovskaya and Bunnett, 2004). There is evidence that PAR-1, which is activated by thrombin and PAR-2, which is activated by trypsin, mast cell tryptase and coagulation factors, have a crucial role in inflammatory diseases of diverse tissues, including the gut, skin and airways (MacFarlane *et al*, 2001; Ossovskaya and Bunnett, 2004). In particular, a possible role of PAR-2 in a rat model of periodontitis involving also prostaglandin release and MMP-2 and MMP-9 activation has been reported (Holzhausen *et al*, 2005). However, no information regarding the distribution and possible role of PARs has been reported in acute or chronic inflammatory conditions of the human oral cavity. The aim of the study was to explore a possible role of PAR-1 and PAR-2 and related bioactive mediators (MMP-2, MMP-9, COX-1, COX-2) in OFG.

Materials and methods

Patients selection

Eighteen patients with OFG were retrospectively identified from the files of the Department of Odontostomatology of the University of Florence and the Department of Dentistry and Surgery of the University of Bari. The series included patients with OCD (seven cases), MRS (six cases), CG (five cases). There were eight women and 10 men. The median age of the patients with OCD was 17 years (range 7–79 years). The median age of the patients with MRS was 49.5 (range 27–67 years). The median age of the patients with CG was 49.4 (range 35–62 years). Upon histopathological revision, two cases of OCD and one case of CG were excluded for inadequate biopsy material and/or clinical data. Therefore, the immunohistochemical investigations were performed on 13 out of 15 remaining cases (five OCD, five MRS and three CG) as well as five cases of normal oral mucosa, taken as control.

Immunohistochemistry

Sections 4 μm in thickness were cut from tissue blocks of formalin-fixed paraffin-embedded tissues. All sections were deparaffinized in Bio-Clear (Bio Optica, Milan, Italy) and dehydrated using graded ethanol. To block endogenous peroxidase activity, the slides were treated with 3.0% hydrogen peroxidase in distilled water for 10 min. Antibodies included: anti-PAR-1 (mouse monoclonal antibody, clone ATAP 2; Zymed laboratories Inc., South San Francisco, CA, USA); anti-PAR-2 (mouse monoclonal antibody, clone SAM 11; Zymed); anti-MMP-2 (mouse monoclonal antibody, clone 42–5D11; Oncogene research products, Cambridge, MA, USA); anti MMP-9 (mouse monoclonal antibody, clone 56–2A4; Oncogene research products); anti-COX-1 (mouse monoclonal antibody, clone COX 111; Zymed); anti-COX-2 (mouse monoclonal antibody, clone COX 229; Zymed).

Antigen retrieval was routinely performed by microwave pretreatment (Microwave MicroMED T/T Mega; Milestone, Bergamo, Italy) in TEC (Tris-EDTA-citrate buffer pH 7.8) for 35 min. After blocking non-specific antigen with normal horse serum (UltraVision, LabVision, Fremont, CA, USA), the sections were incubated with the primary antibodies diluted in Antibody Diluent (Ventana Medical Systems, Tucson, AZ, USA): (i) anti-PAR-1 and PAR-2 diluted 1:50, and incubated for 1 h at room temperature (RT); (ii) anti-MMP-2 and anti-MMP-9 diluted 1:10, and incubated for 1 h at RT; (iii) anti-COX-1 and anti-COX-2 diluted 1:50, and incubated for 2 h at RT. Staining was carried out using a biotin-conjugated anti-rabbit/anti-mouse secondary antibody (Ultra Vision) and streptavidin-peroxidase (Ultra Vision). The bound antibodies were visualized using 3.3′ diaminobenzidine (DAB, BioGenex, San Roman, CA, USA) and aminoethylcarbazol (AEC; LabVision) as chromogen for PARs and diaminobenzidine for MMPs and COXs. Nuclei were lightly counterstained with Mayer's hematoxylin. Negative controls were performed by substituting the primary antibody with a non-immune serum at the same concentration. Control sections were treated in parallel with the samples in the same run.

Immunostained sections were independently assessed by three observers (S.K., D.M., M.S.) and the results were expressed according to semiquantitative criteria as negative staining (score 0), 1–20% (score 1+), 21–50% of positive cells (score 2+), and more than 50% of positive cells (score 3+). The staining intensity was scored on a scale as weak, moderate or strong.

Results

Clinical findings

Oral symptoms in cases of incomplete MRS [oligo and monosymptomatic (CG)] were labial enlargement, oral mucosal swelling, gingival erythematous lesions and fissured tongue. One MRS patient demonstrated facial nerve palsy. Patients with OCD differed from patients with MRS for a cobblestone appearance and/or multiple painful ulcers of the oral mucosa.

Histopathological study

On histopathological examination, mucosal specimens from patients suffering from CG, MRS and OCD showed essentially two patterns of inflammatory reactions: (i) edematous/lichenoid which represents the first phase of the inflammatory process in OFG and in OCD, and (ii) granulomatous which represents the terminal phase of the inflammatory process. The former was seen in three out of four cases of CG, in three out of six MRS cases and in three out of five OCD cases, and was characterized by prominent subepithelial edema, increased number of dilated lymphatic vessels (Figure 1) and a moderate to marked inflammatory infiltrate in lichenoid distribution. The infiltrate was mainly composed of lymphocytes and histiocytes intermingled with numerous plasma cells (Figure 2). The granulomatous pattern of inflammation was observed in one CG case, in three MRS cases and in two OCD cases, and was characterized by the presence of a variable number of non-caseating granulomas, composed of epithelioid

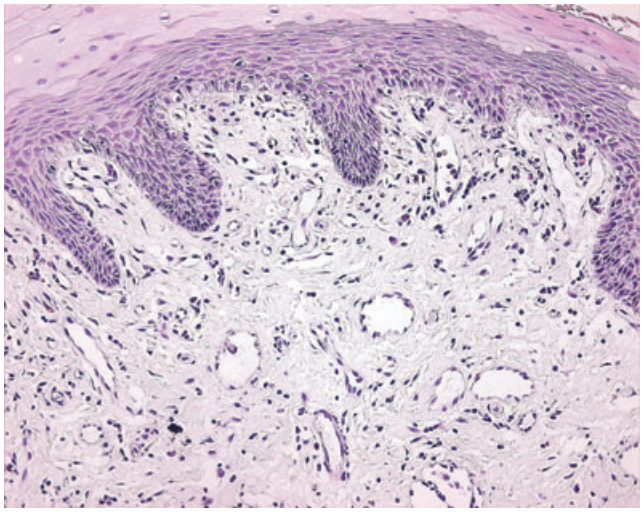

Figure 1 Edematous pattern in orofacial granulomatosis. Marked subepithelial edema, increased number of dilated lymphatic vessels and a slight inflammatory infiltrate within the chorion is observed

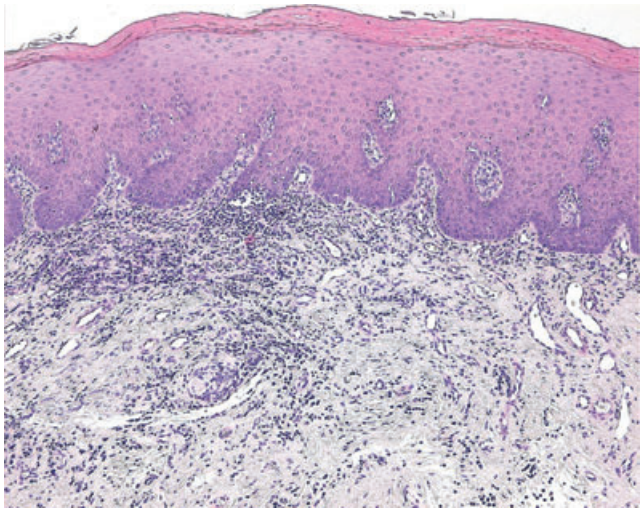

Figure 2 Lichenoid pattern in orofacial granulomatosis. Marked inflammatory infiltrate, mainly composed of lymphocytes and histiocytes in lichenoid distribution

histiocytes intermingled with giant cells, lymphocytes, and plasma cells within the chorion (Figures 3 and 4). Increased numbers of mastocytes were detected in both edematous/lichenoid and granulomatous lesions.

Immunohistochemical analysis

Protease-activated receptor-1 immunohistochemical expression was confined to the cells' cytoplasm, with occasional peripheral membrane pattern, whereas evaluation of PAR-2 immunoreactions displayed predominantly cytoplasmic with occasional cell membrane and nuclear staining. In normal oral mucosa, PAR-1 was expressed in the basal and suprabasal epithelial keratinocytes. PAR-1 positivity was also consistently observed in the pericytes and endothelial cells decorating the

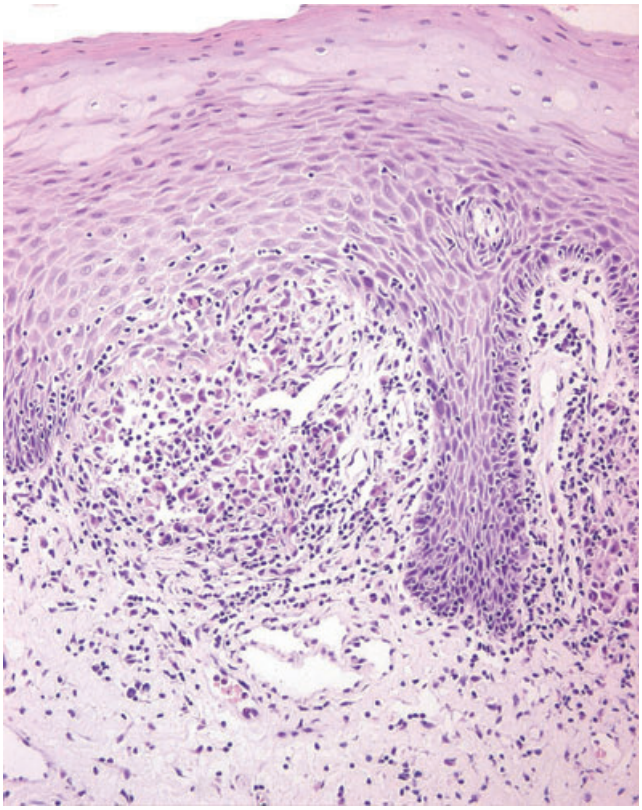

Figure 3 Granulomatous pattern in orofacial granulomatosis. A non-caseating granuloma is present in the subepithelial region

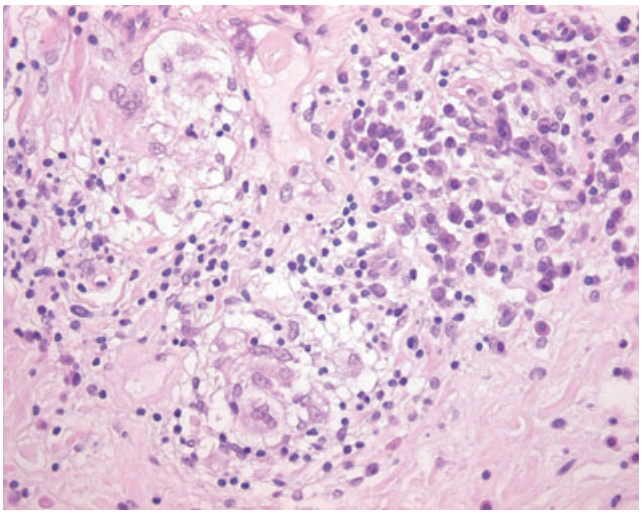

Figure 4 Granulomatous pattern in orofacial granulomatosis. Epithelioid histiocytes intermingled with giant cells, lymphocytes, plasma cells and scattered mastocytes are seen

blood vessels. Scattered fibroblasts were also PAR-1 positive. PAR-2 immunoreactivity was predominantly localized to the basal and suprabasal epithelial layers, whereas the upper superficial layers were unstained. Endothelial cells of the superficial dermal blood vessels, fibroblasts, and mast cells also stained positive.

Results of the immunohistochemical analysis in OFG patients are given in Table 1. Overall, PAR-1 and PAR-2 expressions were related to the intensity of the inflammatory infiltrate. PAR-1 positivity was mostly observed in mononuclear inflammatory cells in lichenoid and edematous lesions whereas a strong PAR-2 immunostaining was detected in the cell cytoplasm of epithelioid histiocytes and giant cells in granulomatous lesions, irrespective of the clinical features (OCD *vs* MRS) (Figure 5). MMP-2 and MMP-9 staining was observed in the cytoplasm of fibroblasts, vascular endothelial cells and inflammatory cells. Stromal staining was poorly defined and gave the impression to be predominantly present in the extra-cellular matrix. MMPs expression was also seen, at varying frequencies and intensities, in the overlying epithelium. In OFG biopsies, MMP-2 and MMP-9 were expressed in the inflammatory cells of the edematous/lichenoid lesions and markedly overexpressed in granulomatous lesions, especially MMP-9 (Figure 6). COX-1 was weakly and variably expressed in both edematous/lichenoid and granulomatous lesions while COX-2 was constantly and markedly expressed in granulomatous lesions, independently from the clinical features [OCD *vs* MRS (oligo and monosymptomatic (CG)] (Figure 7).

Discussion

Our study shows that: (i) PAR-1 and PAR-2 are overexpressed in biopsies taken from OFG patients; (ii) the

Table 1 PAR-1, PAR-2, MMP-2, MMP-9, COX-1, and COX-2 immunohistochemical expression in 13 cases of orofacial granulomatosis

			PAR-1		*PAR-2*		*MMP-2*		*MMP-9*		*COX-1*		*COX-2*	
No.	*Diagnosis*	*Type of inflammation*	*Infl. cells*	*Epit. cells*	*Infl. cells*	*Epit. cells*	*Infl. cells*	*Epit. cells*	*Infl. cells*	*Epit. cells*	*Infl. cells*	*Epit. cells*	*Infl. cells*	*Epit. cells*
1	MRS	Edematous-lichenoid	1+	1+	1+	2+	2+	3+	2+	3+	1+	2+	2+	2+
2	MRS	Edematous-lichenoid	1+	1+	1+	1+	2+	2+	2+	2+	1+	2+	2+	3+
3	MRS	Granulomatous	3+	1+	3+	2+	1+	1+	2+	2+	1+	1+	2+	2+
4	MRS	Edematous-lichenoid	3+	1+	2+	3+	2+	2+	3+	3+	2+	2+	3+	3+
5	MRS	Granulomatous	2+	1+	3+	2+	3+	3+	3+	3+	1+	3+	3+	3+
6	CG	Granulomatous	2+	1+	3+	1+	3+	3+	3+	3+	2+	2+	3+	2+
7	CG	Edematous-lichenoid	1+	1+	1+	2+	2+	3+	2+	3+	1+	2+	2+	2+
8	CG	Edematous-lichenoid	3+	1+	2+	1+	2+	2+	2+	3+	1+	1+	1+	2+
9	Crohn	Edematous-lichenoid	2+	2+	1+	3+	1+	3+	1+	2+	1+	2+	2+	3+
10	Crohn	Granulomatous	3+	3+	3+	3+	3+	3+	3+	3+	2+	3+	3+	3+
11	Crohn	Edematous-lichenoid	2+	1+	1+	1+	2+	2+	2+	2+	1+	2+	2+	2+
12	Crohn	Edematous-lichenoid	2+	1+	1+	2+	2+	3+	3+	3+	1+	2+	3+	3+
13	Crohn	Granulomatous	1+	2+	3+	1+	2+	2+	3+	2+	1+	1+	2+	2+

MRS, Melkersson–Rosenthal syndrome; CG, cheilitis granulomatosa; Infl., inflammatory; Epit., epithelial; 1+, 1–20% of positive cells; 2+, 21–50% of positive cells; 3+, more than 51% of positive cells.

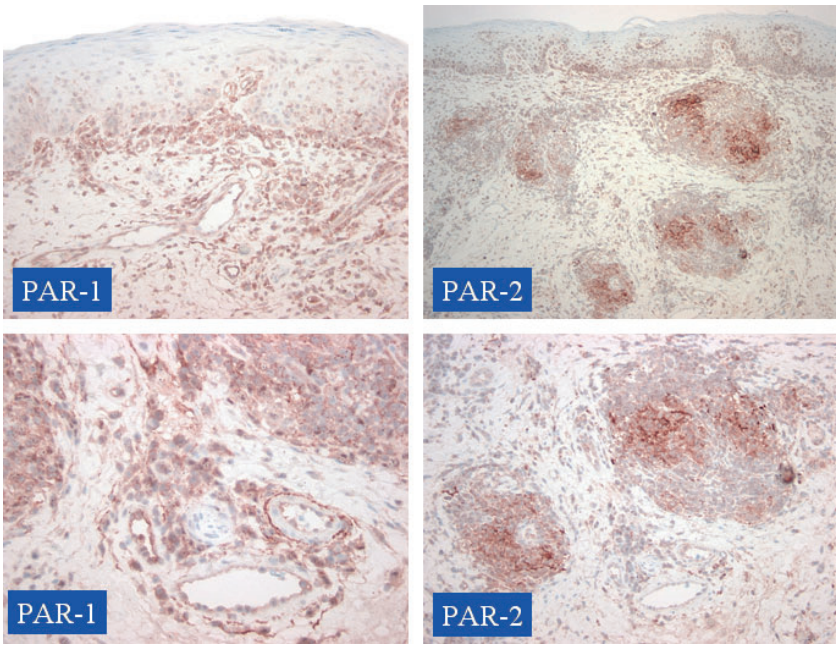

Figure 5 PAR-1 positivity is observed in mononuclear inflammatory cells in lichenoid and edematous lesions. Strong PAR-2 immunostaining is detected in the cytoplasm of epithelioid histiocytes and giant cells in granulomatous lesions

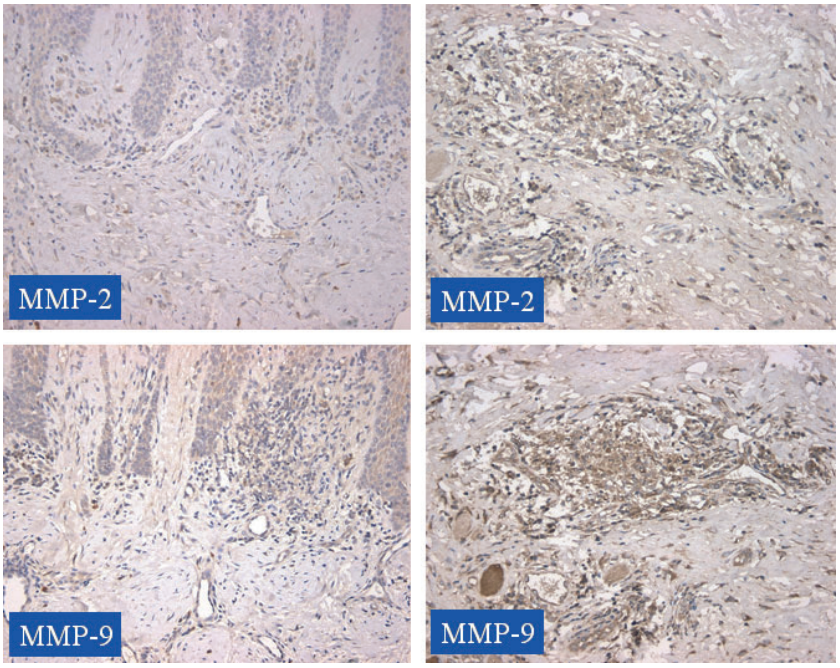

Figure 6 MMP-2 and MMP-9 are expressed in the inflammatory cells of edematous/lichenoid lesions and markedly overexpressed in granulomatous lesions, especially MMP-9

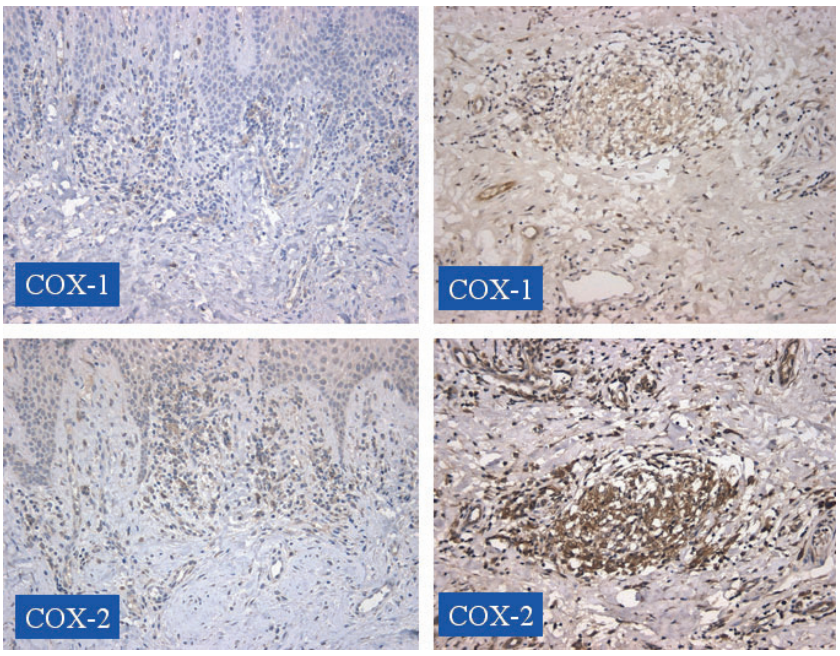

Figure 7 COX-1 is weakly and variably expressed in edematous/lichenoid lesions while COX-2 is constantly and markedly expressed in granulomatous lesions

intensity and distribution of receptor expression is associated with the histopathological pattern (edematous/lichenoid *vs*. granulomatous) but not to the clinical type of the lesion (MRS *vs*. OCD); (iii) the intensity of receptor expression is also related to the grade of the inflammatory response; (iv) MMP-2, MMP-9, COX-2 are also upregulated in OFG biopsies. PAR-2 immunoreactivity was markedly overexpressed in granulomatous lesions while immunostaining for PAR-1 was mostly observed in the mononuclear cell inflammatory component in early lichenoid/edematous lesions, in a manner independent from the clinical diagnosis. Thus, PARs overexpression seems to occur at the site of the oral granulomatous inflammatory disorder, irrespective from the lesion type: idiopathic *vs* secondary or localized *vs* systemic.

The present immunohistochemical investigation shows that PAR-1 and -2 are present on virtually all the cells of oral tissues derived from OFG patients, including inflammatory cells (monocytes-macrophages, lymphocytes) that are resident in or recruited to the oral tissues, surface epithelial cells, mast cells, vascular

endothelial cells, and fibroblasts. All these cell types may contribute to the process of inflammation in such a microenvironment. A marked up-regulation of PAR-2 was demonstrated in macrophages localized within granulomatous foci. Intense PAR-2 immunoreactivity in these areas was, however, patchy in distribution, indicating a mixed population of PAR-2-reactive cells. It is not possible to determine which protease(s) activates PARs in the context of OFG tissues. Factors of the coagulation cascade may contribute to PARs activation as proteases released from mast cells or proteases originating from other cellular sources. Present findings that a large number of mast cells were seen in both edematous/lichenoid and granulomatous OFG lesions suggest a major role of PAR-2 activating proteases, e.g. tryptase, in OFG tissues.

We provided evidence for a simultaneous overexpression of PARs, MMPs and COXs, suggesting that these receptors and enzymes could synergistically contribute to the proinflammatory mechanism of OFG. MMP-2 and MMP-9 were found largely expressed by the stromal fibroblasts as well as by inflammatory cells recruited in oral tissues. However, because immunohistochemistry cannot discriminate between latent and activated forms of MMPs (Curran and Murray, 1999), we cannot conclude that a significant MMP proteolytic activity is present in the OFG tissue. A recent study has proposed a role of PAR-2 in periodontitis in rats through a mechanism involving prostaglandin release and MMP-2 and MMP-9 activation (Holzhausen *et al*, 2005). Treatment with the PAR-2 agonist, SLIGRL-NH2 when compared with the inactive peptide LRGILS-NH2, increased alveolar bone destruction and granulocytic infiltration in gingival tissues, and caused significant over-expression of COX-1, COX-2, MMP-2 and MMP-9 (Holzhausen *et al*, 2005). Evidence for the involvement of MMPs and COXs in PAR-2 agonist-induced periodontitis was confirmed by a reduced inflammatory response following doxycycline (which inhibits MMP-2 and MMP-9 expression) and indomethacin (which induces non-selective inhibition of COX-1 and COX-2) administration (Holzhausen *et al*, 2005).

A possible interaction between the MMP family and PARs has been recently proposed in diverse tumoral models. Activation of PAR-1 or PAR-2 increased the levels of MMP-2 and MMP-9 activity in prostate cancer cell lines, favoring their potential metastatic role (Wilson *et al*, 2004). More recent findings (Boire *et al*, 2005; Pei, 2005) have suggested that interstitial collagenase MMP-1, mainly secreted by peritumoral stromal fibroblasts, functions as a protease agonist of PAR-1 cleaving and activating the receptor to promote cell migration and metastasis. Further research will clarify whether MMP-1 may contribute to PAR-1 activation also in the context of inflammatory, non-tumoral, conditions.

Overexpression of PARs in OCD is in line with the previous observations indicating that PAR-1 expression is up-regulated in biopsies taken from patients with inflammatory bowel diseases (IBD) (Vergnolle *et al*, 2004). In the colon of IBD patients PAR-1 could be activated not only by proteases from the coagulation cascade, such as thrombin, factor Xa, or factor VIIa, but also by bacterial proteases (Vergnolle *et al*, 2004). In the ileal and colonic mucosa of IBD patients, an increased number of mast cells has been demonstrated accompanied by elevated histamine and tryptase levels, suggesting that tryptase, as PAR-2 agonist, may be involved in the pathogenesis of IBD (He, 2004).

The present findings of PAR-1 and PAR-2 overexpression related to the intensity and type of inflammatory response support the hypothesis that pharmacological blockade of PARs may be beneficial in these types of chronic oral inflammatory disorders. Because it is possible that PARs-induced oral inflammation involves prostaglandin release and MMP activation, also drugs that block these enzymatic activities may contribute positively to the treatment of these diseases.

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